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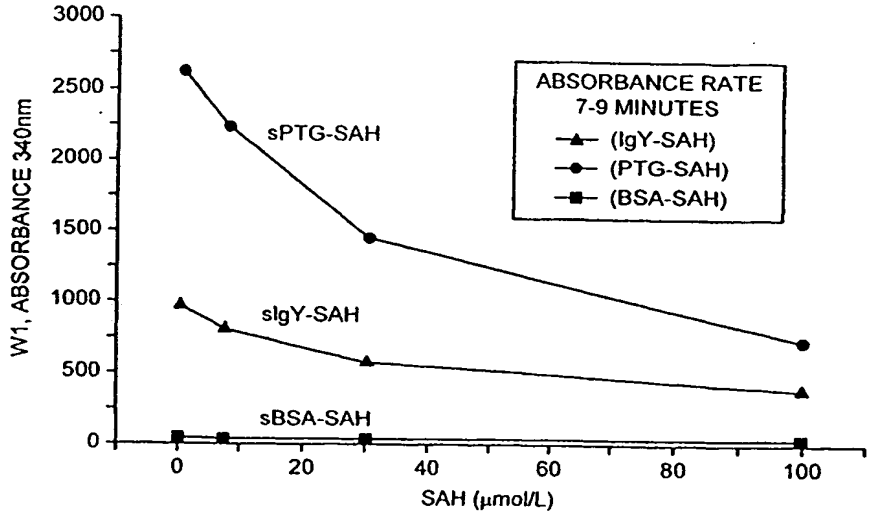

(57) Abstract

The invention provides a method for assaying homocysteine in a sample, said method comprising: contacting said sample with two or three stable aqueous reagents containing a polyhapten, a homocysteine converting enzyme, a primary antibody capable of binding to said polyhapten whereby to produce a complex, and if desired one or more of a co-substrate for said homocysteine converting enzyme, a reducing agent, a further enzyme capable of converting said co-substrate or a conversion product of said homocysteine converting enzyme, and a second antibody capable of binding to said complex, said primary antibody also being capable of binding to said co-substrate or a conversion product of one of said enzymes whereby the quantity of said complex produced is indicative of the content of homocysteine in said sample; and photometrically detecting said complex.

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Assay for Homocysteine

The invention relates to an assay for homocysteine in body fluids or fluids derived therefrom and to kits for such assays.

Homocysteine is a sulphur-containing amino acid that is closely related to methionine and cysteine. There is no DNA-coding for homocysteine and it is not present in naturally occurring proteins. Homocysteine is formed in the metabolism of the essential amino acid methionine. In plasma about 1% of homocysteine exists in the free reduced form, about 70% is bound to albumin and the rest is present in low molecular weight disulphides, predominantly with cysteine. Total homocysteine, a term describing all molecular forms of homocysteine (e.g. free and bound) is abbreviated hereinafter as tHcy. In the literature the term homocysteine is often abbreviated as Hcy and used to describe both free and bound homocysteine.

Hyperhomocysteinemia is generally defined as fasting plasma tHcy above 15 μmol/L. It has been shown to be a risk factor for cardiovascular disease and for complications in pregnancy and congenital malformations. A link between impaired homocysteine metabolism and neuropsychiatric disorders and cognitive impairment in the elderly has also been shown.

Normalisation of homocysteine levels may be achieved by life-style changes (e.g. quitting smoking, taking exercise, reducing coffee consumption, improving diet, etc.) or by vitamin supplementation.

Accordingly there has been significant emphasis in recent years on the development of assays for homocysteine. Such assays are available for example from Abbott Laboratories (US), Axis-Shield (UK and NO), Bio-Rad (NO), and A/C Diagnostics (US). Such assays are moreover described in patent applications from Axis-

Shield (NO) and the University of Glasgow (UK), e.g. US-A-5631127 and WO98/07872.

Direct determination of homocysteine is not straightforward and these assays generally involve enzymatic conversion of homocysteine and direct or indirect determination of a homocysteine conversion product. Thus for example US-A-5631127 describes conversion of homocysteine and adenosine to S-adenosyl homocysteine (SAH) using SAH-hydrolase and determination of SAH, while WO98/07272 describes the conversion of homocysteine and water to α-ketobutyrate and hydrogen sulphide using homocysteine desulphurase and determination of either the α-ketobutyrate or the hydrogen sulphide.

For homocysteine to be available as an analytical parameter for most clinical laboratories however, a homocysteine assay is needed which may be performed in the commercially available automated clinical chemistry analysers, sometimes referred to as clinical chemistry platforms, for example the systems available from Hitachi and Roche Diagnostics.

Generally clinical chemistry analysers are characterised by the use of simple photometric measurements, e.g. colorimetric, turbidimetric or nephelometric measurements. Additionally the number of manual handling steps for running assays on these analysers have to be as few as possible. Preferably little or no sample pre-treatment should be required and ideally the reagents used should be assay-ready in a stable form. The number of compartments for reagents in these analysers is typically extremely limited, and since the analysers may be arranged to determine several different analytes in a sample, often only two or three reagent compartments may be available for an assay for a single analyte.

Furthermore, the total assay time required per sample must be relatively short both to optimise

throughput and to avoid disturbing the instrument logistics and hence the instrument's handling of different analytes.

The existing homocysteine assays mentioned above are not generally applicable to a range of automated analysers, in particular since the reagents are not sufficiently few in number and in ready-to-use stable form. Thus for example the Enzymatic Homocysteine Assay Kit from A/C Diagnostics has three buffer solutions and four reagents which have to be dissolved in the buffers before use. In the Abbott IMx system, four reagents are required - these are all kept in separate compartments and cannot, due to stability and incompatibility problems, be mixed or combined to reduce the total number of reagents. The Shield Homocysteine Chromogenic Assay likewise uses four reagents.

We have now found that, using a polyhapten, a homocysteine converting enzyme and an antibody, it is possible to produce an immunoassay for homocysteine which requires only two, or at most three, stable, ready-to-use reagents.

Viewed from one aspect therefore the invention provides a method for assaying homocysteine in a sample, said method comprising:

contacting said sample with two or three stable aqueous reagents containing a polyhapten, a homocysteine converting enzyme, a primary antibody capable of binding to said polyhapten whereby to produce a complex, and if desired one or more of a co-substrate for said homocysteine converting enzyme, a reducing agent, a further enzyme capable of converting said co-substrate or a conversion product of said homocysteine converting enzyme, and a secondary antibody capable of binding to said complex, said primary antibody also being capable of binding to said co-substrate or a conversion product of one of said enzymes whereby the quantity of said complex produced is indicative of the content of

homocysteine in said sample; and photometrically detecting said complex.

The assay of the invention may be used to determine tHcy or free homocysteine; in the former and more usual case the sample will generally be treated with a reducing agent to liberate bound homocysteine.

By "stable" it is meant that the aqueous reagents should be capable after storage at 20°C for at least 3 months, preferably at least 6 months, more preferably at least 18 months, of being used to determine homocysteine content in a 10 μmol/L L-homocysteine standard with a loss in accuracy of no more than 10% preferably no more than 5% as compared with freshly prepared equivalent reagents. In testing stability, determination of the antibody:polyhapten complex may be performed turbidimetrically using a Hitachi 911 apparatus.

Viewed from a further aspect the invention also provides a homocysteine assay reagent kit comprising two or three stable aqueous reagents containing a polyhapten, a homocysteine converting enzyme, a primary antibody capable of binding to said polyhapten whereby to produce a complex, and if desired one or more of a co-substrate for said homocysteine converting enzyme, a reducing agent, a further enzyme capable of converting said co-substrate or a conversion product of said homocysteine converting enzyme, and a secondary antibody capable of binding to said complex, said primary antibody also being capable of binding to said co-substrate or a conversion product of one of said enzymes.

In the method of the invention, the homocysteine content of the sample, preferably the tHcy value, is determined indirectly by determining the amount of the polyhapten:antibody complex, preferably by nephelometry or turbidimetry. The homocysteine content may be determined quantitatively, e.g. in absolute units such as μmol/L, or alternatively the determination may be

qualitative, e.g. simply that it is above a predetermined threshold such as 15, 18 or 20 μmol/L. Generally, the assay measurement will be calibrated against standard homocysteine solutions containing known concentrations of homocysteine, usually L-homocysteine; however for assays run on automated analysers only occasional calibration will be necessary, e.g. when reagent reservoirs are refilled.

Where an antibody is used in the assay method and kits of the invention, this may be a polyclonal or more preferably a monoclonal antibody; however the term antibody is also used to cover single chain antibodies, antibody fragments (e.g. Fab fragments), and oligopeptides and oligonucleotides capable of highly specific binding to the polyhapten or polyhapten:primary antibody complex. Appropriate such specific binding oligonucleotide and oligopeptides may be identified by conventional combinatorial chemistry techniques, e.g. using phage display libraries. Antibodies to the hapten in the polyhapten, ie. to the direct or indirect homocysteine conversion product of the homocysteine conversion enzyme, the co-substrate, or to the polyhapten: primary antibody complex may also be raised in a conventional fashion, e.g. by mouse immunization followed by fusion with myeloma cells, etc.

In the assay method of the invention, the aqueous reagents are preferably contacted with the sample sequentially with the analyte determination taking place after addition of the final reagent. However, unlike earlier assays, if the time of determination is carefully controlled, determination may take place before complex generation is complete: in this way the time required for performing the assay may be reduced, e.g. to less than 15 minutes, advantageously less than 10 minutes and especially advantageously less than 5 minutes.

The sample investigated using the method of the

invention may be any homocysteine containing sample. Generally it will be a body fluid or a liquid derived from a body fluid, e.g. blood, urine, cerebrospinal fluid, serum or plasma. Serum is preferred and plasma is particularly preferred. Where the sample is one in which homocysteine is protein bound (e.g. blood, serum, plasma, etc.) it is preferred that one of the reagents, preferably the first reagent, added to the sample, should contain a reducing agent so as to liberate the free reduced homocysteine. Dithiothreitol (DTT) and tris(2-carboxyethyl)phosphine (TCEP) are preferred as reductants. TCEP is especially preferred since it is a strong reductant which is stable at pH values of about 7. Other pH 7 stable reductants could however be used if desired.

The first reagent also preferably contains the co-substrate for the homocysteine converting enzyme, if such a co-substrate is required. Thus for example where the homocysteine converting enzyme is SAH-hydrolase it is desirable to include adenosine or an adenosine analog in the reagents.

In general one or more of the reagents will preferably contain an agent which promotes precipitation of the polyhapten:primary antibody complex. Suitable such materials include polysaccharides, polyhydroxyl compounds, carbohydrates, alginates, chitosan, heparin, heparinoids, poloxamers and poloxamines. Especially preferred are polyalkylene oxides such as polethylene glycol (PEG). PEG of molecular weight 1 to 10 kD, particularly 2 to 8 kD and more particularly about 6 kD, functions particularly well in this regard.

Besides agents such as PEG, complex precipitation may be enhanced by reducing pH to acid levels. This however is less preferred.

Less preferably, immunocomplex precipitation may be enhanced using organic solvents or salts, e.g. ammonium acetate, metal cations, 2-ethyl-6,9-diaminoacridine

lactate, protamine, polyacrylates, etc.

Since agents which promote complex precipitation may also promote precipitation of other proteins present in the sample, where such agents are used they are preferably present in two or all three of the reagents.

Depending on whether two or three reagents are used according to the invention, the different components may be distributed differently in the reagents. For a two reagent system, the first reagent desirably contains an immunocomplex precipitation promoter (e.g. PEG), a co-substrate (e.g. adenosine) if such is necessary, a reductant (e.g. TCEP), and a polyhapten (e.g. poly-SAH), while the second reagent desirably contains the primary antibody (e.g. anti-SAH), the homocysteine converting enzyme (e.g. SAH-hydrolase), a complex precipitation promoter (e.g. PEG) and, if desired, the secondary antibody. Where three reagents are used, two or three preferably contain an immunocomplex precipitation promoter (e.g. PEG); while the primary antibody and the homocysteine converting enzyme are preferably not formulated together with the polyhapten or, where present, the co-substrate.

If desired the reagents used may include a carrier protein, e.g. casein or albumin, both as a stabilizer and to compensate for variations in the background signal from proteins in the sample (e.g. plasma proteins) which may occur at varying levels in different samples - by adding carrier protein the percentage variation between samples in the proteins responsible for background signal is reduced.

In the assay method of the invention, an analyte competes with the polyhapten for binding to the primary antibody. The analyte may be a homocysteine co-substrate or a homocysteine conversion product or (where a second enzyme is used) a conversion product of any of these. The polyhapten preferably is a water-soluble compound or compound mixture which has at least two,

preferably at least 5, more preferably at least 10 and especially preferably at least 50 hapten moieties which are the same as or similar to at least part of the analyte such that the primary antibody is capable of binding both to the analyte and the hapten moieties of the polyhapten. More generally, the polyhapten preferably has at least one hapten moiety per 100 kD of its molecular weight and the polyhapten preferably has a molecular weight of at least 500 kD, more preferably at least 1 MD, e.g. up to 3 MD. Conveniently, it has a polymeric backbone structure onto which the haptens are bound, e.g. a polypeptide structure (for example a protein such as a thyroglobulin or a polypeptide such as polyaspartic acid), a polysaccharide (such as a dextran derivative, alginic acid and mannan), or a synthetic polymer with pendant functional groups such as hydroxyl, carboxyl and/or amino groups. Porcine thyroglobulin, which has a molecular weight of 660 kD, is especially preferred since its size is appropriate and since it can be dimerized to produce a larger polyhapten. In general proteins and polypeptides will be preferred as polyhapten backbone materials since their isoelectric points, and hence the precipitation characteristics of the polyhapten:primary antibody complex, can readily be modified. The hapten moieties may be coupled to the polyhapten backbone using conventional chemistry, e.g. using peptide coupling between amine and carboxyl groups of the hapten and the polyhapten-backbone compound.

Thus, by way of example, SAH can be coupled to porcine thyroglobulin using EDC/EDAC (1-ethyl-3-(dimethylaminopropyl)-carbodiimide) or another carboxyl activator, optionally after treating the protein with succinic acid. A one-pot reaction is possible, but it is preferred to succinylate the protein in a separate step before conjugation to SAH. Succinic acid couples to free amino groups on the protein, provides a pendant carboxyl for SAH attachment and also lowers the

isoelectric point of the protein. The carboxyl activator permits coupling of SAH carboxyls with protein amino groups as well as of SAH amino groups with native and succinic acid-deriving carboxyls on the proteins. In such a reaction, N-hydroxy succinimide may also be used to promote SAH:protein coupling.

As mentioned above, the polyhapten is preferably water-soluble. However it is also possible to use colloidal particles, for example nano-particles, e.g. particles having sizes in the 1 to 1000 nm, especially 50 to 800 nm range, as a matrix onto which the haptens are bound. The particle size should be such that the particles form a stable aqueous dispersion from which they do not settle out. The particles may be of a synthetic polymer with suitable functional groups for hapten attachment.

The homocysteine converting enzyme used in the assay method may be any enzyme capable of directly or indirectly using or generating an antigenic analyte. Typical enzymes include SAH-hydrolase, homocysteinase (homocysteine desulphurase), methionine synthase, cystathionine β-synthase, and betaine-homocysteine methyl transferase. Where the enzyme used is SAH-hydrolase, the analyte will generally be adenosine or SAH or an enzymic conversion product of one of these; however SAH is the preferred analyte. Where the enzyme used is homocysteine desulphurase, the analyte will generally be an enzyme conversion product of α-ketobutyrate.

In the assay method of the invention, a secondary antibody which binds to the polyhapten:primary antibody complex may be used if desired. In this way precipitation of the polyhapten:primary antibody complex is enhanced and the sensitivity of the assay may be improved. The secondary antibody may for example be an anti-mouse IgG polyclonal antibody where the primary antibody is a mouse antibody. The secondary antibody

may be polyclonal or monoclonal. The secondary antibody can be of any origin, e.g. rabbit, chicken, etc., as long as its cross-reactivity with components in the sample under investigation is not such as to cause any change in light transmission or scattering. Thus cross-reactivity with human plasma proteins and antibodies is desirably absent.

The secondary antibody need not be a traditional antibody: any specific binding material which enhances precipitation of the polyhapten:primary antibody complex may be used, e.g. antibody fragments, oligopeptides, oligonucleotides, single chain antibodies, etc., optionally with two or more coupled together.

The use of secondary antibodies however potentially increases the compatibility problems, ie. the problems faced in producing an assay method requiring only two or three reagents. For a three reagent system, it is possible to have primary and secondary antibodies and polyhapten in separate reagents. However, it is possible to formulate the primary and secondary antibodies together if a chaotropic salt is also included in the reagent. Such chaotropic salts include for example salts containing SCN^-, CCl_3COO^-, CF_3COO^-, Cs^+, Li^+, or Mg^{2+} ions and guanidine chloride.

The twin antibody reagent will preferably also contain a reagent which promotes precipitation of the immunocomplex, e.g. a polyalkylene oxide such as PEG or other such compounds as discussed earlier. Thus the antibodies, PEG and chaotropic salt will be at a higher concentration than in the reaction medium when they are mixed with sample and polyhapten. By combining a small volume of this antibody containing reagent with a larger volume of another solution, e.g. another reagent mixed with the sample, dilution of the chaotropic salt will permit immunoprecipitation to begin. This novel strategy can be used in other assays to obtain signal enhancement and forms a further aspect of the invention.

Viewed from this aspect, the invention provides an aqueous assay reagent containing a chaotropic salt, an immuno-precipitation enhancer (e.g. PEG), a primary antibody and a secondary antibody.

The invention will now be illustrated further by reference to the following non-limiting Examples and to the accompanying drawings, in which:

Figure 1 shows a plot of absorption against time for calibration samples under the assay system of Example 8;
Figure 2 shows a plot of absorption against time for calibration samples under the assay system of Example 9;
Figure 3 shows dose response curves for the assays using the polyhaptens of Examples 4 and 5;
Figure 4 shows a comparison of immunoprecipitation signal obtained using BSA-SAH, IgY-SAH and PTG-SAH conjugates under the assay conditions of Example 8;
Figure 5 shows a plot of absorption against time for calibration samples under the assay system of Example 10;and
Figure 6 shows the dose response curve for the assay of Example 10.

EXAMPLE 1

Primary Antibody Production

Balb/c mice were immunised with SAH coupled to bovine serum albumin. After the fourth injection, splenocytes from the mice were fused with myeloma cells as described by Gaffre et al. "Preparation of Monoclonal Antibodies: Strategies and Procedures", Meth. Enzymology pages 31-46 (1987). The hybrid cell supernatants were screened for binding to SAH and BSA and those binding to SAH but not BSA were selected for cloning. After stabilising the clone, the cells were grown *in vitro* in hollow fibre systems and the antibodies were purified from the

supernatant using Protein A-sepharose column chromatography (see Ey et al. Immunochem. 15: 429-436 (1978)).

EXAMPLE 2

Secondary Antibody Production

Commercially available polyclonal anti-mouse IgG antibodies may be used. These are produced by injecting mouse IgG into another species (e.g. chicken, goat, rabbit, etc), harvesting antisera and purifying the harvested antisera by immunoaffinity chromatography using antigens coupled to agarose gels.

In the following Examples the secondary antibody is LAJD-112 from La Jolla Diagnostics Inc, California.

(The secondary antibody may alternatively be a monoclonal antibody against the polyhapten:primary antibody complex. In this event it may be produced analogously to the antibody of Example 1 using in place of the SAH-bovine serum albumin conjugate the polyhapten:primary antibody complex or a fragment thereof including the binding site between the polyhapten and the primary antibody).

EXAMPLE 3

Polyhapten production

(A) 150 μL succinic anhydride (7.5 mg/mL in N,N-dimethylformamide) was added dropwise to 5 mL of porcine thyroglobulin (PTG) (5 mg/mL in 10 mM phosphate buffer, pH 7.4). The mixture was allowed to react for 4 hours at 20-25°C with constant stirring.

(B) The product of step (A) was dialysed against 2L of 2-morpholinoethanesulphonic acid (MES) buffer, pH 5.5.

(C) 10 mL of the modified PTG from step (B) (approx. 5 mg/mL in the MES buffer) was mixed with 6.8 mL SAH solution (17 mg in 6.8 mL MES buffer) and 0.7 mL sulpho-N-hydroxysuccinimide solution (5 mg in 1 mL MES buffer).

(D) With constant stirring, 7.6 mg (1-ethyl-3(3-dimethylaminopropyl)carbodiimide.HCl (EDC) was added and the mixture was allowed to react at 20-25°C for 3-5 hours.

(E) The product of step (D) was dialysed against 5L of 10 mM phosphate buffer, pH 7.4. The PTG-polySAH product is used as a polyhapten.

Bovine serum albumin-SAH polyhapten (BSA-SA-SAH) is produced by succinylation followed by carbodiimide coupling using water soluble or insoluble carbodiimides, e.g. EDC/EDAC, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide-p-toluene sulphonate, or dicyclohexylcarbodiimide, IgG-SAH and IgY (chicken immunoglobulin)-SAH polyhaptens are produced analogously. Dextran-SAH polyhapten is prepared by reaction of amino-dextran with succinic anhydride followed by SAH coupling using a carbodiimide. Alginic acid-SAH is prepared by carbodiimide coupling analogously to steps (D) and (E) in Example 3.

EXAMPLE 3

Polyhapten production

(A) 120 µl succinic anhydride (7.5 mg/mL in N,N-dimethylformamide (DMF)) is added dropwise to 1 ml of poly-L-glutamic acid (10 mg/mL dissolved in 10 mM

phosphate buffer, pH 7.4). The mixture is allowed to react for 4 hours at 20-25°C with constant stirring.

(B) The resulting modified (N-terminal blocked) polyglutamic acid is dialysed against 1L of MES buffer, pH 5.5.

(C) 1.6 ml of the dialysed product (approx. 7 mg/ml in MES buffer) is mixed with 0.8 ml SAH solution (1.9 mg/mL in 100 mM MES Buffer, pH 5.5) and 0.8 ml sulpho-N-hydroxysuccinimide solution (0.5 mg in 1 mL 100 mM MES buffer, pH 5.5).

(D) With constant stirring, 22 mg EDC is added. The mixture is allowed to react for 3 to 5 hours at 20-25°C.

(E) The product (s-poly-Glu-SAH) is dialysed against 3L of 10 mM phosphate buffer, pH 7.4.

EXAMPLE 5

Polyhapten production

(A) 150 µl succinic anhydride (7.5 mg/ml in DMF) is added dropwise to 2 ml of casein (5 mg/ml in 10 mM phosphate buffer, pH 7.4). The mixture is allowed to react for 4 hours at 20-25°C with constant stirring.

(B) The resulting modified casein is dialysed against 1L of MES buffer, pH 5.5.

(C) 1 ml of the modified casein (approx. 4 mg/ml in 100 mM MES buffer, pH 5.5) is mixed with 0.5 ml SAH solution (2.5 mg/mL in 100 mM MES Buffer, pH 5.5) and 0.07 ml sulpho-N-hydroxysuccinimide solution (5 mg in 1 mL 100 mM MES buffer, pH 5.5).

(D) With constant stirring, 1.2 mg EDC is added. The mixture is allowed to react for 3 to 5 hours at 20-25°C.

(E) The product (s-casein-SAH) is dialysed against 3L of 10 mM phosphate buffer, pH 7.4.

EXAMPLE 6

Polyhapten preparation

(A) 150 μl succinic anhydride (100 μmol/ml in DMF) is added dropwise to 2 ml of IgY (chicken antibody, 46 mg/ml in 10 mM PBS-buffer, pH 7.4). The mixture is allowed to react for 4 hours at 20-25 °C with constant stirring.

(B) The resulting modified IgY is dialysed against 10mM MES-buffer, pH 5.5.

(C) 1 ml of the modified IgY (approx. 4 mg/ml in 100 mM MES buffer, pH 5.5) is mixed with 0.5 ml SAH solution (2.5 mg/mL in 100 mM MES Buffer, pH 5.5) and 0.07 ml sulpho-N-hydroxysuccinimide solution (5 mg in 1 mL 100 mM MES buffer, pH 5.5).

(D) With constant stirring, 1.2 mg EDC is added. The mixture is allowed to react for 3 to 5 hours at 20-25°C.

(E) The product (sIgY-SAH) is dialysed against 3 L of 10mM PBS-buffer.

EXAMPLE 7

Polyhapten preparation

(A) 1 mL of 0.1M $NaIO_4$ is added dropwise to 1 ml mannan (5 mg/ml in water). The mixture is allowed to react for

30 minutes at 20-25°C with constant stirring.

(B) The modified mannan is dialysed against 1L of 10 mM carbonate-buffer, pH 9.5.

(C) 1 mL of the modified mannan (approx. 4 mg/ml in 10 mM carbonate buffer, pH 9.5) is mixed with 1 ml SAH solution (1.9 mg/ml in 10 mM carbonate buffer, pH 9.5). The temperature is maintained at 2-10°C and the reaction is allowed to proceed for 10 hours.

(D) 20 µl 0.1M aqueous $NaBH_4$ is added and the mixture is incubated at 20-25°C for 3 hours.

(E) The mannan-SAH conjugate is dialysed against 1.5L of 10 mM phosphate buffer, pH 7.4.

Figure 4 of the accompanying drawings shows a comparison of the immunoprecipitation signal detected using sBSA-, sIgY-, and sPTG-SAH conjugates as the polyhapten. As can be seen, the precipitation properties of the different conjugates do vary.

The immunoprecipitation ability of the polyhaptens of the Examples is PTG-poly-SAH > sIgY-SAH > mannan-SAH > BSA-SA-SAH = s-poly-Glu-SA > s-Casein-SAH > alginic acid-SAH.

EXAMPLE 8

Two Reagent Assay

Reagent 1

10 mM phosphate buffer*, 0.15M NaCl, pH 7.4	4800 µL
30% w/v PEG 6000	1200 µL
10% Triton X-100 (aqueous)	350 µL

2 mM Adenosine (in phosphate buffer*)	15 μL
50 mM TCEP (aq)	140 μL
PTG-SAH polyhapten (2mg/mL)	400 μL

Reagent 2

10 mM phosphate buffer*	730 μL
30% w/v PEG 6000	270 μL
Primary antibody (3 mg/mL)	400 μL
SAH hydrolase (≈ 350 U/mL)	600 μL

Assay Protocol

10 μL sample (e.g. human plasma) is mixed with 180 μL of Reagent 1 and incubated for 0.25 to 5 minutes at 37°C. 50 μL of Reagent 2 is added, the mixture is incubated for 3 to 10 minutes at 37°C and the precipitation signal is determined by absorption measurements at 340-450 nm, preferably 340 nm.

The assay is calibrated with L-homocyst(e)ine standards.

The total assay time can be as little as 3 minutes. Figure 1 of the accompanying drawings shows a plot of absorption vs time for calibration samples. The jump at measuring point 12 marks the addition of Reagent 2. The assay time may be shortened by careful timing of Reagent addition and absorption measurement and using the difference between measurement at two times after addition of Reagent 2 (e.g. measuring points 15 and 21 in Figure 1). This may be done even though the signal strength may not have reached it maximum at the later point.

EXAMPLE 9

Two Reagent Assay

Reagent 1

As Example 8

Reagent 2

10 mM phosphate buffer*	625 µL
30% w/v PEG 600	270 µL
Primary antibody (3 mg/mL)	400 µL
SAH hydrolase (≈ 350 U/mL)	600 µL
5M NaSCN (aq.)	70 µL
Secondary antibody (rabbit-anti-mouse) (2 mg/mL)	35 µL

Assay Protocol

As for Example 8

Fig. 2 of the accompanying drawings shows a plot of absorption vs. time for a series of calibration samples.

The dose response curve for the assays using the polyhaptens of Examples 4 and 5 are shown in Figure 3 of the accompanying drawings. These are obtained by plotting the absorption difference (dA 340 nm) between the measurements at measuring points 15 and 21 (ie. 7.5 and 10.5 minutes).

EXAMPLE 10

Two Reagent Assay

Reagent 1

10 mM Bis-Tris, pH 6.4	190 µL
Triton X-100 (aqueous)	50 µL
0.5 mM Adenosine (aqueous)	128 µL

5 mM TCEP in Bis-Tris pH 6.4	60 µL
water	233 µL
1M NaSCN (aqueous)	50 µL
1.5 M NaCl (aqueous)	79 µL
PTG-SAH polyhapten (≈ 2mg/mL)	210 µL

Reagent 2

10% PEG (aqueous)	900 µL
100 mM phosphate-buffer, pH 7.4	210 µL
water	140 µL
1.5 M NaCl	220 µL
SAH hydrolase (≈ 350 U/mL)	230 µL
Primary antibody (3 mg/mL)	300 µL

Assay Protocol

10 µL sample (e.g. human plasma) is mixed with 50 µL of Reagent 1 and incubated for 5 minutes at 37°C. 200 µL of Reagent 2 is added, the mixture is incubated for 5 minutes at 37°C and the precipitation signal is determined by absorption measurements at 340-450 nm, preferably 340 nm.

The assay is calibrated with L-homocyst(e)ine standards.

Figure 5 of the accompanying drawings shows a plot of absorption vs time for calibration samples. Dose response curves can be derived from the signal difference between points over the final 5 minutes of the reaction. The dose response curve shown in Figure 6 is derived by plotting the absorption difference between points 13 and 18 as a function of calibrator concentration.

Claims:

1. A method for assaying homocysteine in a sample, said method comprising:

contacting said sample with two or three stable aqueous reagents containing a polyhapten, a homocysteine converting enzyme, a primary antibody capable of binding to said polyhapten whereby to produce a complex, and if desired one or more of a co-substrate for said homocysteine converting enzyme, a reducing agent, a further enzyme capable of converting said co-substrate or a conversion product of said homocysteine converting enzyme, and a second antibody capable of binding to said complex, said primary antibody also being capable of binding to said co-substrate or a conversion product of one of said enzymes whereby the quantity of said complex produced is indicative of the content of homocysteine in said sample; and

photometrically detecting said complex.

2. A method as claimed in claim 1 wherein at least one of said reagents contains a said secondary antibody.

3. A method as claimed in claims 1 or 2 wherein said complex is determined nephelometrically or turbidimetrically.

4. A method as claimed in any of claims 1 to 3 wherein photometric determinatination takes place before complex generation is complete.

5. A method as claimed in any of claims 1 to 4 wherein said sample is a a serum or plasma sample.

6. A method as claimed in any of claims 1 to 5 wherein at least one of said reagents additionally

contains an agent which promotes precipitation of said complex.

7. A method as claimed in claim 6 wherein said agent which promotes precipitation is polyethylene glycol.

8. A method as claimed in any of claims 1 to 7 wherein at least one of said reagents further comprises a carrier protein.

9. A method as claimed in any of claims 1 to 8 wherein said polyhapten consists of a backbone structure onto which the haptens are bound.

10. A method as claimed in claim 9 wherein said backbone structure is porcine thyroglobulin.

11. A method as claimed in any of claims 1 to 10 wherein the hapten of said polyhapten is S-adenosine homocysteine (SAH).

12. A method as claimed in any of claims 1 to 11, wherein at least one of said reagents contains said primary and secondary antibodies and additionally contains a chaotropic salt.

13. A homocysteine assay reagent kit comprising two or three stable aqueous reagents containing a polyhapten, a homocysteine converting enzyme, a primary antibody capable of binding to said polyhapten whereby to produce a complex, and if desired one or more of a co-substrate for said homocysteine converting enzyme, a reducing agent, a further enzyme capable of converting said co-substrate or a conversion product of said homocysteine converting enzyme, and a second antibody capable of binding to said complex, said primary

antibody also being capable of binding to said co-substrate or a conversion product of one of said enzymes.

14. A kit as claimed in claim 13 wherein at least one of said reagents contains a said secondary antibody.

15. A kit as claimed in either of claims 13 or 14, wherein at least one of said reagents additionally contains an agent which promotes precipitation of said complex.

16. A kit as claimed in claim 15 wherein said agent which promotes precipitation is polyethylene glycol.

17. A kit as claimed in any of claims 13 to 16 wherein at least one of said reagents further comprises a carrier protein.

18. A kit as claimed in any of claims 13 to 17 wherein said polyhapten consists of a backbone structure onto which the haptens are bound.

19. A kit as claimed in claim 18, wherein said backbone structure is porcine thyroglobulin.

20. A kit as claimed in any of claims 13 to 19, wherein the hapten of said polyhapten is S-adenosine homocysteine (SAH).

21. A kit as claimed in any of claims 13 to 20 wherein at least one of said reagents contains said primary and secondary antibodies and additionally contains a chaotropic salt.

22. A kit as claimed in any of claims 13 to 21

containing two said reagents.

23. An aqueous assay reagent containing a chaotropic salt, an immuno-precipitation enhancer, a primary antibody and a secondary antibody.

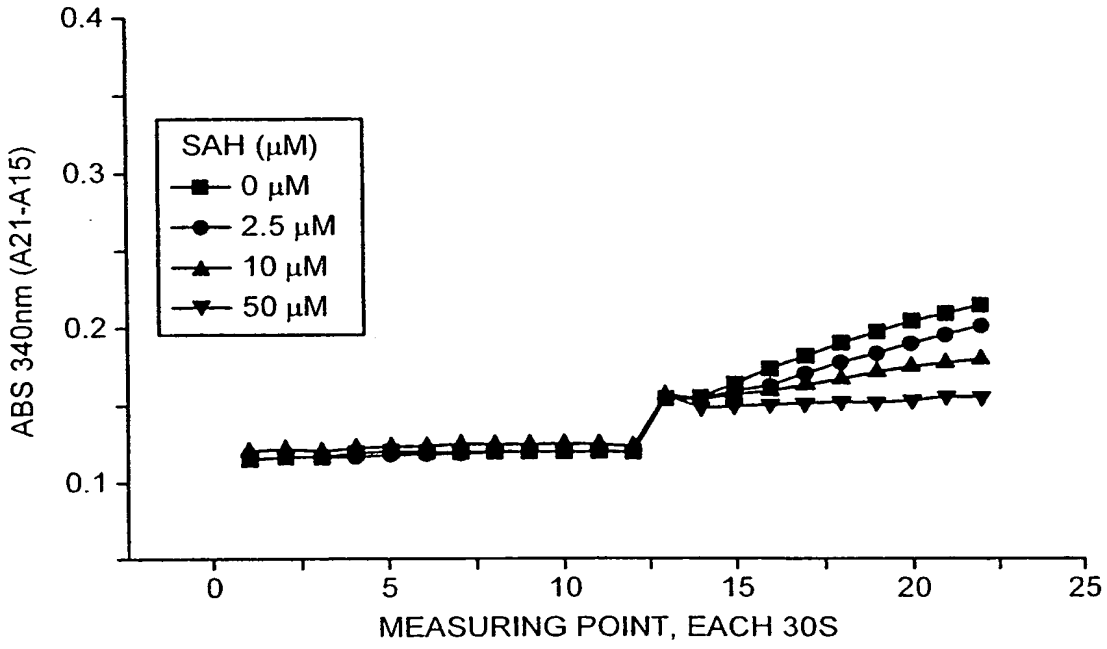

FIG. 1

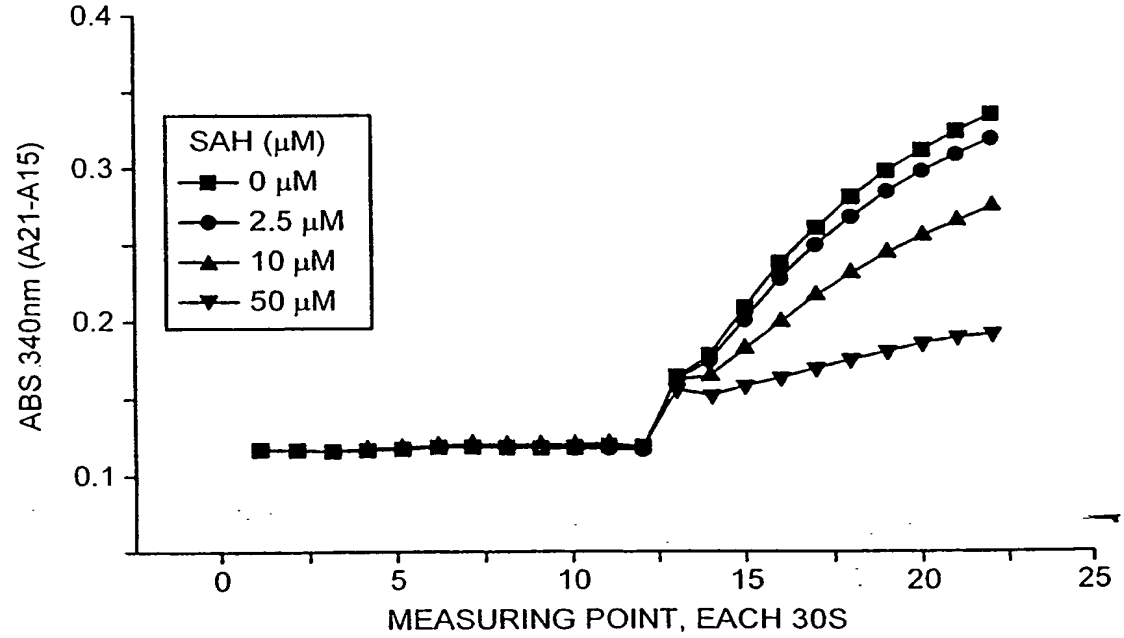

FIG. 2

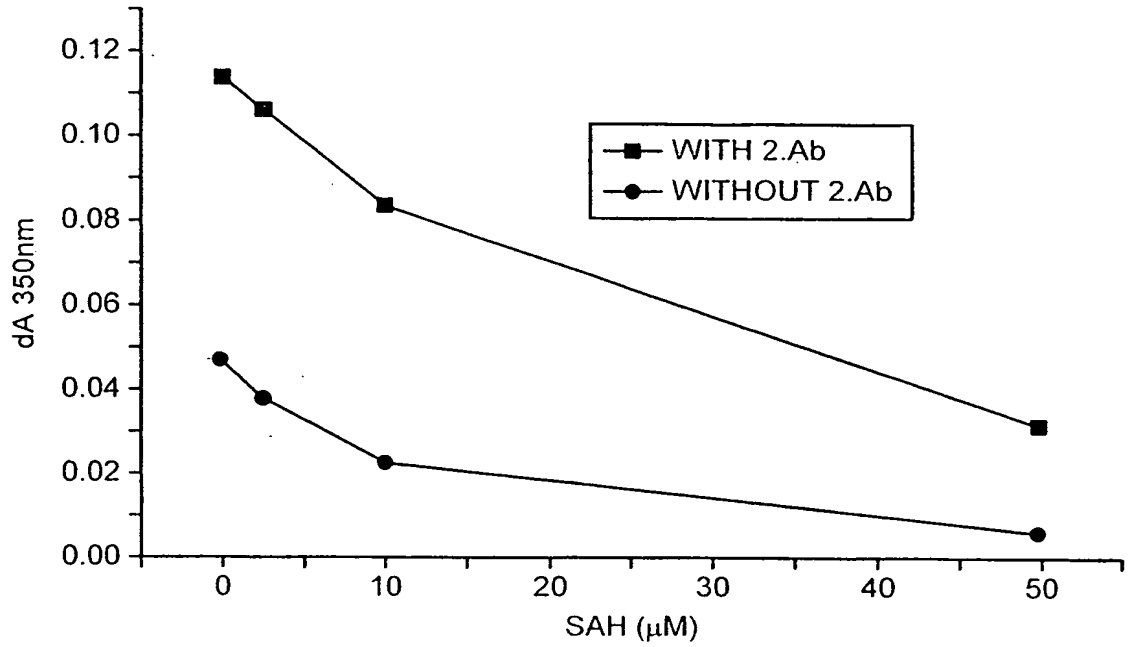

FIG. 3

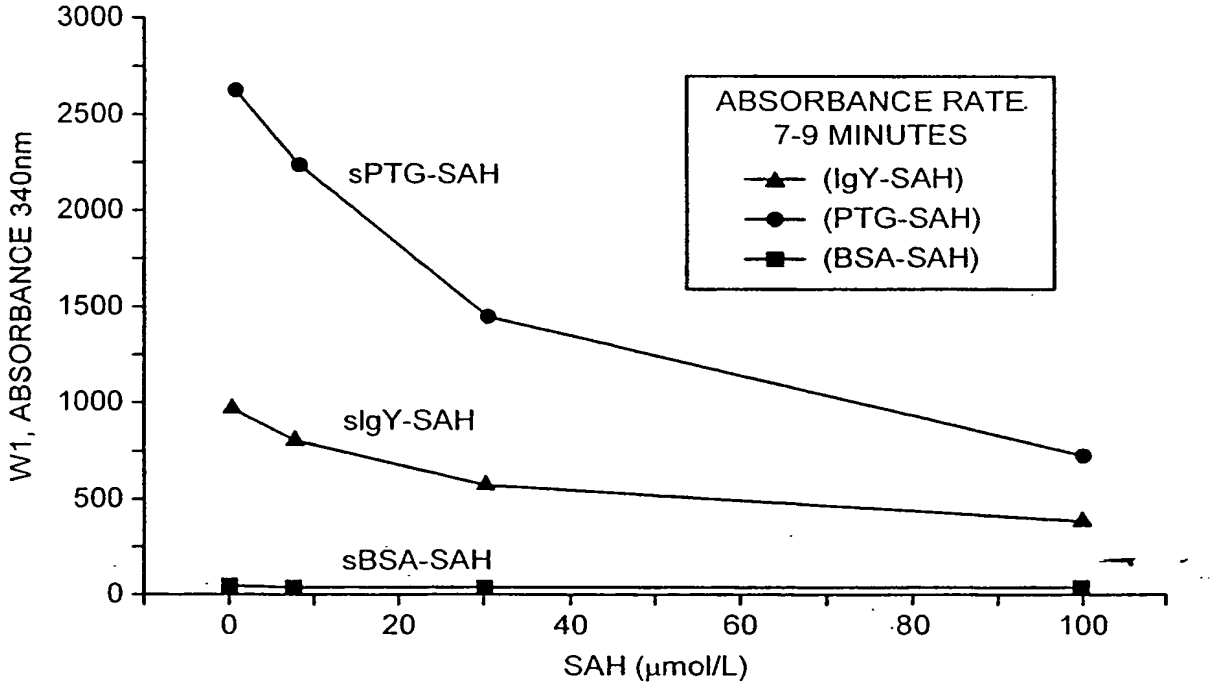

FIG. 4

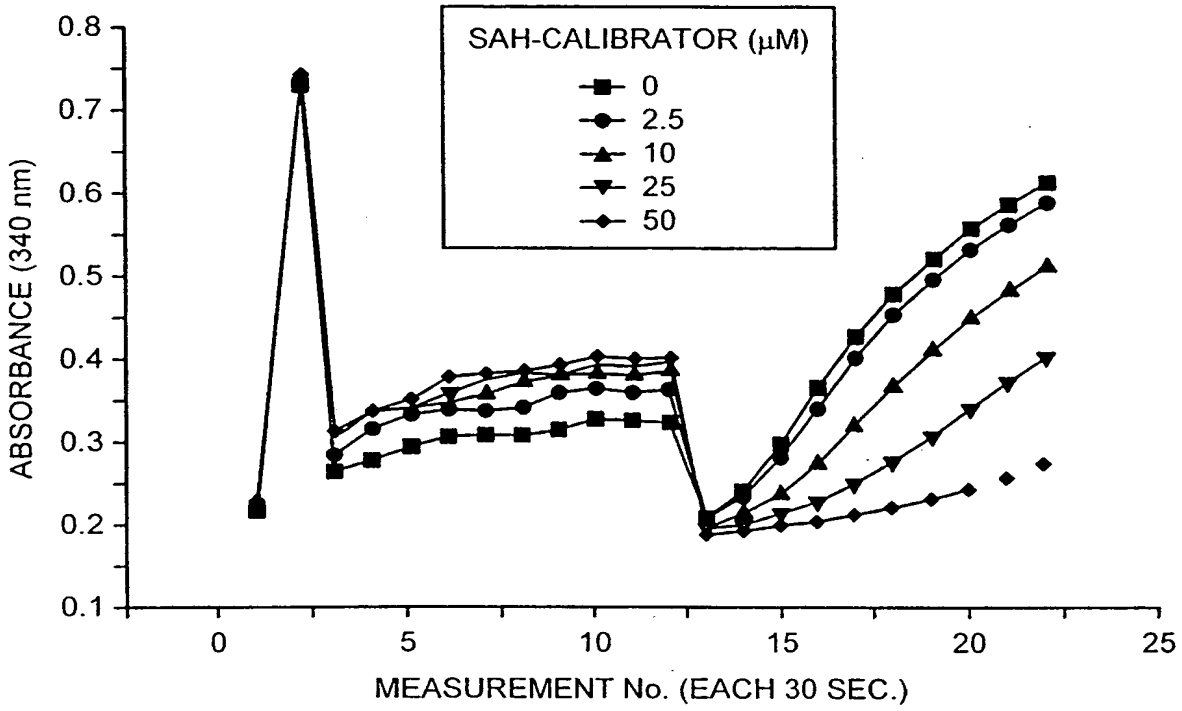

FIG. 5

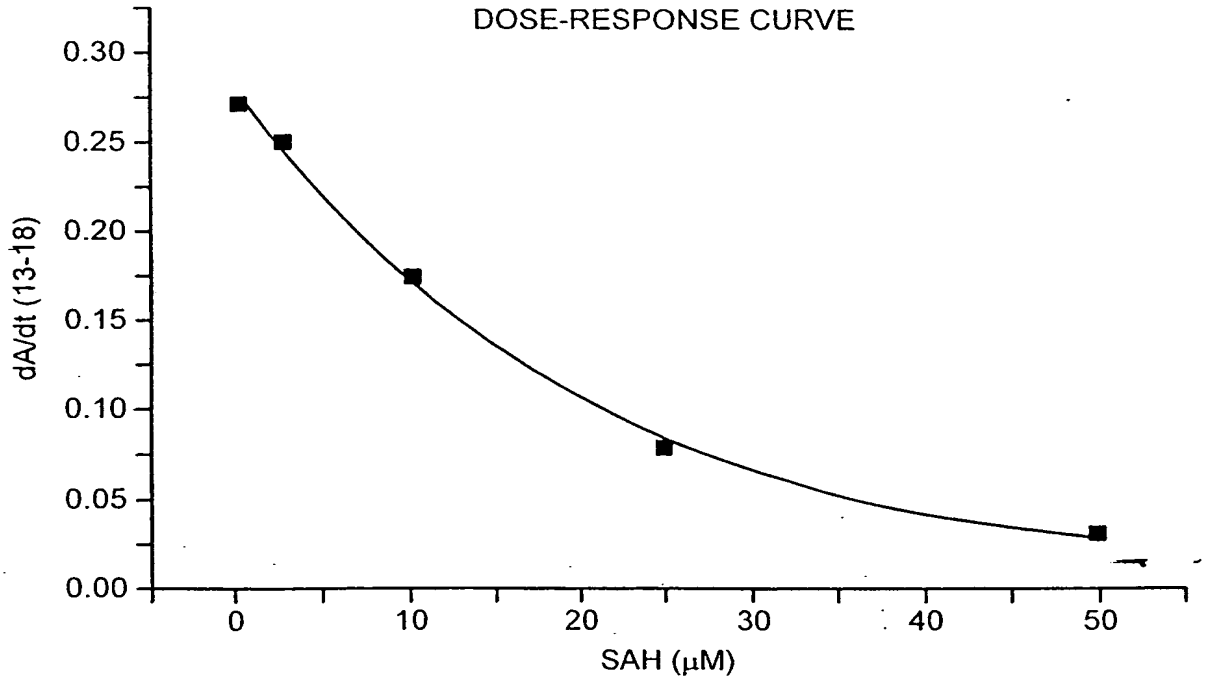

FIG. 6

INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/GB 99/04442

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 726 322 A (AXIS BIOCHEMICALS AS) 14 August 1996 (1996-08-14) page 12, line 21 - line 40; claims 1-3	1-23
A	WO 93 15220 A (COCKBAIN JULIAN R M ;AXIS RESEARCH (NO)) 5 August 1993 (1993-08-05) page 14 page 24, paragraph 4 -page 25, paragraph 2	1-23

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search	Date of mailing of the international search report
14 April 2000	26/04/2000

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Information on patent family members

Int: ional Application No
PCT/GB 99/04442

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0726322 A	14-08-1996	AT 142271 T	15-09-1996
		AU 676480 B	13-03-1997
		AU 4340893 A	01-09-1993
		BR 9305780 A	18-02-1997
		CA 2128512 A	05-08-1993
		CZ 9401763 A	15-12-1994
		DE 69304511 D	10-10-1996
		DE 69304511 T	23-01-1997
		DK 623174 T	13-01-1997
		EP 0623174 A	09-11-1994
		ES 2094524 T	16-01-1997
		FI 943462 A	14-09-1994
		WO 9315220 A	05-08-1993
		GR 3021365 T	31-01-1997
		HU 67550 A	28-04-1995
		JP 2870704 B	17-03-1999
		JP 8506478 T	16-07-1996
		NO 942729 A	15-09-1994
		SK 87894 A	12-04-1995
		US 5958717 A	28-09-1999
		US 5631127 A	20-05-1997
		US 5827645 A	27-10-1998
WO 9315220 A	05-08-1993	AT 142271 T	15-09-1996
		AU 676480 B	13-03-1997
		AU 4340893 A	01-09-1993
		BR 9305780 A	18-02-1997
		CA 2128512 A	05-08-1993
		CZ 9401763 A	15-12-1994
		DE 69304511 D	10-10-1996
		DE 69304511 T	23-01-1997
		DK 623174 T	13-01-1997
		EP 0623174 A	09-11-1994
		EP 0726322 A	14-08-1996
		ES 2094524 T	16-01-1997
		FI 943462 A	14-09-1994
		GR 3021365 T	31-01-1997
		HU 67550 A	28-04-1995
		JP 2870704 B	17-03-1999
		JP 8506478 T	16-07-1996
		NO 942729 A	15-09-1994
		SK 87894 A	12-04-1995
		US 5958717 A	28-09-1999
		US 5631127 A	20-05-1997
		US 5827645 A	27-10-1998